Short report: Choline plasma levels are related to mRNA Nrf2 expression in chronic kidney disease?

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ABSTRACT

Background & Aims: Choline is an amine osmolyte with antioxidant potential. A limited number of studies have implied that choline modulates the nuclear factor-erythroid 2-related factor 2 (Nrf2) pathway, a major cytoprotectant system. However, there are no data regarding such an interaction in patients with chronic kidney disease (CKD). This cross-sectional pilot study therefore aimed to evaluate the possible relationship between choline plasma levels and transcriptional expression of Nrf2 in patients with CKD on hemodialysis (HD). **Methods:** This study was performed in 24 HD patients [54 ± 10 years, 14 men, BMI 26.4 ± 4.5 kg/m^2]. Choline plasma levels were measured by LC-MS/MS. Nrf2 mRNA expression was measured by real-time quantitative polymerase chain reaction (rt-PCR) in isolated peripheral blood mononuclear cells (PBMC). **Results:** We used Pearson's correlation (rho) to determine the correlations with Nrf2 expression and observed a positive correlation between choline plasma levels and Nrf2 (rho=0.56, *P*=0.004). **Conclusions:** This finding suggests that choline may play a role in Nrf2 expression in CKD.

Keywords: chronic kidney disease, choline, nuclear factor-erythroid 2-related factor 2.

Introduction

Choline is a quaternary ammonium compound found in a range of foodstuffs, ranging from wheat germ to animal sources, such as liver, red meat, fish, and egg yolk [1]. The choline content in some animal foods, according to the United States Department of Agriculture [2], is 169 mg in 1 egg (50.3g), 160 mg in 1 steak (284g), and 49.3 mg in fish fillet (116g). In 1998, the Institute of Medicine recognized choline as a nutrient, which the adequate intake (AI) recommendation is 425 mg/d for women and 550 mg/d for men aged older than 19 [3]. After ingestion, more than 50% of the choline is converted to phosphatidylcholine, also called lecithin or sphingomyelin (components of the cell and mitochondrial membranes). Choline can be oxidized to the osmolyte betaine by the gut microbiota, or in the inner membrane of mitochondria by choline dehydrogenase. Betaine is a major methyl donor in the homocysteine and methionine cycle and has an important role in regulating cell volume. Choline is also a precursor of the neurotransmitter acetylcholine [4–6].

Besides the established actions of choline, a negative association between high choline intake and inflammation has been reported [7–9]. A model of airway disease in the mouse has already shown that choline supplementation reduced oxidative stress and the inflammatory response [10]. Indeed, a recent finding has confirmed that increased choline intake (three eggs/day for four weeks) reduced cytokine plasma levels in patients with metabolic syndrome [11]. A further *in vitro* study has shown that when bovine mammary epithelial cells were heat stressed after being treated with choline, oxidate damage levels were reduced, while concomitantly superoxide dismutase (SOD) and glutathione peroxidase (GSH-PX) levels were increased due the activation of the cytoprotective protein kinase R-like endoplasmic reticulum kinase/nuclear factor erythroid 2-related factor 2 (PERK/Nrf-2) pathway [12]. In support of these findings, another study in vascular smooth muscle cells has shown that choline treatment again caused Nrf2 translocation to the nucleus and subsequent increase in heme-oxygenase-1 (HO-1) and (NAD(P)H Quinone Dehydrogenase-1 (NQO-1) levels [13].

Persistent inflammation and oxidative stress are essential components of the phenotype of many burdens of the age-related burden of lifestyle diseases, such as chronic kidney disease (CKD). As Nrf2 is vital for cellular defenses against oxidative stress, it has been hypothesized that depressed expression of Nrf2 plays a significant role in the progression and risk for development of complications in CKD [14,15]. As depressed Nrf2 mRNA expression is a common finding in CKD [16] and dietary changes can manipulate choline levels, we performed a cross-sectional pilot study to explore the association between choline plasma and Nrf2 mRNA levels in patients with CKD on hemodialysis (HD).

Methods

Recruitment of Participants

The Ethics Committee of the School of Medicine – Universidade Federal Fluminense approved the study. Twenty-four patients with CKD on HD obtained from our biorepository were involved in this pilot study. Patients who used catheters, were less than 18 years old, had cancer or autoimmune disease, were smokers, were living with HIV, had liver disease, or were in use of catabolic drugs were excluded. Dialysis duration was 3.0–4.5 hours per session, three times per week, with a blood flow greater than 250 mL/min and a dialysate flow of 500 mL/min. Body mass index (BMI) was evaluated.

Sample analysis, Choline plasma analysis, and Nrf2 Expression

Morning blood samples were collected for each patient after overnight fasting. After collection, blood samples were separated into plasma (15 min, 3000 x g, 4 °C) and peripheral blood mononuclear cells (PBMC) and stored in -80 °C until analysis.

Choline plasma levels were measured by LC-MS/MS utilizing a protocol outlined in [17]. Interleukin-6 (IL-6) was analyzed by enzyme-linked immunosorbent assay (ELISA) (Boster Biological Technology, Pleasanton CA, USA). High sensitivity serum C-reactive protein (hsCRP) level was determined using BioClin® kits using the Bioclin BS-120 Chemistry Analyze biochemical analyzer.

Peripheral blood mononuclear cells (PBMC) were obtained from whole blood. The mRNA expressions of Nrf2 and NQO1 were evaluated from PBMC using real-time quantitative polymerase chain reaction (qPCR) (Cardozo et al., 2016) [18]. TaqMan gene expression assay (Thermo Fisher®) was used to detect mRNA expression of Nrf2 (Hs00975961_g1), NQO1 (Hs00168547_m1), and the control gene, GAPDH (Hs02758991_g1). The Prism 7500 Sequence Detection System ABI (Applied Biosystems®) and standard cyclic conditions were used for PCR amplification. Nrf2 and NQO1 mRNA expression were normalized against GAPDH, and the expression level was calculated using the $2^{-\Delta\Delta CT}$ (threshold cycle) method.

Statistical Analysis

Pearson's correlation (rho) was used to determine the correlations with Nrf2 expression. Baseline patient numerical continuous variables were expressed as the median and interquartile range (IQR), or the difference between 75^{th} and 25^{th} percentiles. Categorical

variables were expressed as absolute and relative proportions. Skewed continuous variables were log-transformed when applicable. A statistical significance was set at the level of $P < 0.05$. Statistical analyses were conducted in R, version 4.1.1.

Results

The overall value for choline plasma levels was 11.4 (3.6) ng/uL. **Table 1** shows the baseline characteristics of all patients and stratified by median choline plasma levels.

Table 1. Baseline patient characteristics overall and stratified by median choline plasma levels.

Parameters	Overall	Low Choline (n=10)	High choline (n=14)	*P* value
Sex (M/F)	14/10	6/4	8/6	-
Age (years)	54 ± 10	55 ± 11	52 ± 10	0.61
HD vintage (months)	48 (43)	48 (42)	45.5 (54)	0.991
BMI (kg/m^2)	25.5 (5.6)	26.7 (4.6)	24.7 (4.5)	0.292
Albumin (mg/dL)	4 (0.5)	4.1 (0.6)	4 (0.3)	0.707
Potassium (mg/dL)	4.5 (1)	4.5 (0.8)	4.5 (0.9)	0.432
Urea (mg/dL)	144 (53.7)	141 (54)	149 (48)	0.967
Hemoglobin (mg/dL)	11 (2.7)	11.3 (3.3)	10.9 (1.9)	0.272
IL-6 (mg/dL)	21.6 (19.5)	20.4 (13.7)	23.6 (25.9)	0.353
CRP (mg/dL)	3.9 (7.3)	6.6 (8.4)	3.1 (4.6)	0.018
MDA (nmol/mL)	2.8 (5.9)	2.6 (4.66)	4.6 (6.1)	0.311
mRNA Nrf2	0.9 (0.8)	0.7 (0.6)	1.6 (1.6)	0.009
mRNA NQO1	0.9 (0.7)	0.7 (0.6)	1.1 (0.8)	0.096

Note: Data are presented as mean ± SD, or median (IQR). **Abbreviations:** HD: hemodialysis; BMI: body index mass; IL-6: interleukin-6; CRP:C-reactive protein; MDA: Malondialdehyde; Nrf2: nuclear factor-erythroid 2-related factor 2; NQO1: (NAD(P)H Quinone Dehydrogenase-1.

Figure 1 shows correlation between Nrf2 mRNA expression and choline plasma levels in HD patients **(A)** (rho=0.56, p-value=0.004) and as expected, there was a low positive correlation between mRNA Nrf2 and NQO1 expressions (rho=0.47, p-value=0.008) (**B**). We observed a negative correlation between Nrf2 expression and interleukin-6 (IL-6) (rho=0.47, p-value=0.003 (**C**) and CRP plasma levels in HD patients (**D**) (rho=0.59, p-value=0.01)**.** Choline plasma levels **(Figure 2)**, were negatively correlated with CRP plasma levels (rho=0.45, p-value=0.04).

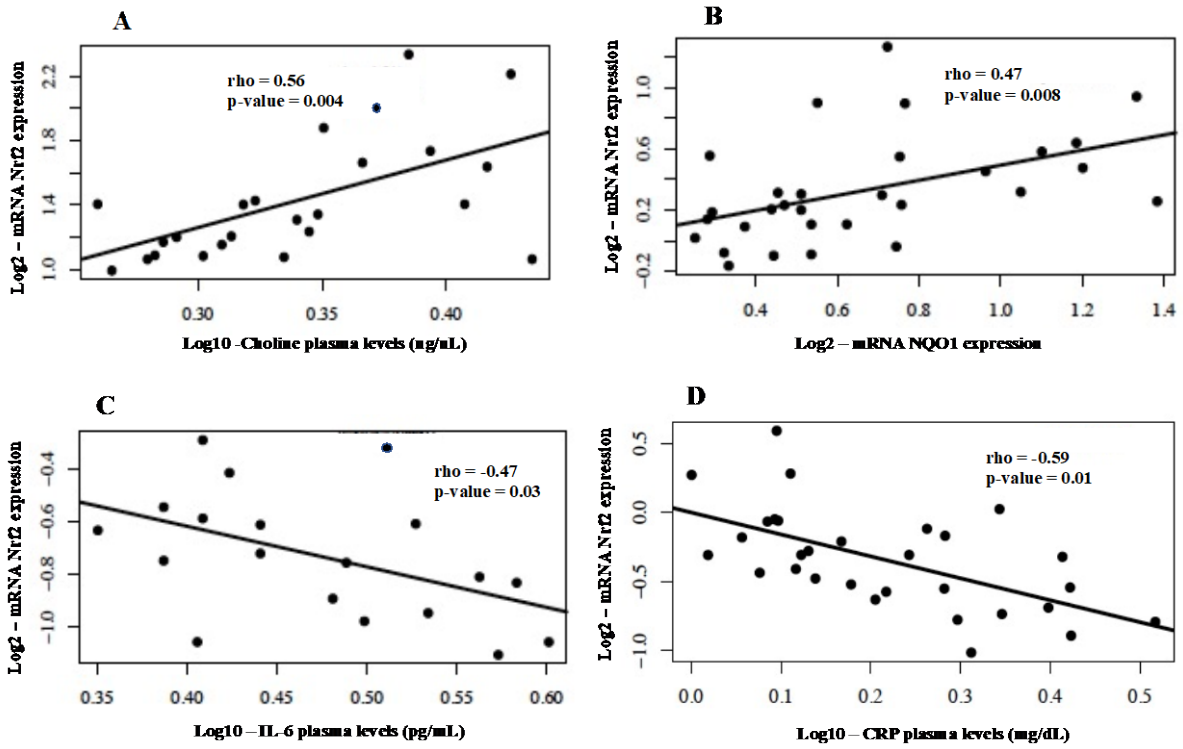

Figure 1. Correlation between Nrf2 mRNA expression and choline plasma levels (A), NQO1expression (B), interleukin-6 (IL-6) (C) and C-reactive protein (CRP) (D) plasma levels in HD patients.

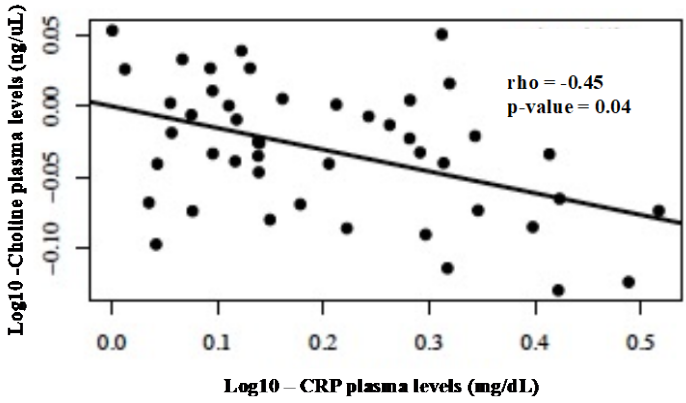

Figure 2. Correlation between C-reactive protein (CRP) and choline plasma levels in HD patients.

Discussion

This cross-sectional pilot study in HD patients demonstrates that Nrf2 expression is positively correlated with choline plasma levels and negatively with inflammatory burden. The clinical implication of this finding is that nutritional strategies that increase choline levels

may have anti-inflammatory and anti-oxidative effects. Clinical nutritional trials with choline-rich food are needed to test this hypothesis.

Nrf2 is a major regulatory factor for over 300 cytoprotective genes. Consequently, it has an important role in mitigating inflammation and oxidative stress via increasing the synthesis of antioxidants enzymes, reducing the expression of NF-kB, and reducing the production of cytokines [15]. As patients with CKD exhibit downregulation of Nrf2 expression, this may lead to an exacerbation of the effects of oxidative stress and inflammation [16,19]. We have clearly demonstrated in this study, a negative correlation between Nrf2 and IL-6 in the patients on HD.

As stimulation of Nrf2 has been shown to slow the progression of kidney disease [15] much recent interest has focused on the possibility of targeting Nrf2 with drugs and bioactive nutrients, such as sulforaphane, resveratrol and curcumin [20–22].

Choline is an essential nutrient for acetylcholine and phospholipid synthesis, and recent studies have also shown its role as an antioxidant [10,11]. According to IOM (Institute of Medicine), the plasma levels can range between 7 and 20 μmol/L, but it is not a good choline status since the levels do not reduce 50% of normal (even under fasting) [23].

Reduced choline levels are associated with the cholinergic pathway and inflammation in Alzheimer's disease [24] and insulin resistance and type-2 diabetes [25]. Moreover, a methionine-choline deficient diet induces non-alcoholic fatty liver disease due to mitochondrial dysfunction and oxidative stress and reduces the expression of Nrf2 [26,27]. Our finding of an independent correlation between choline levels and Nrf2 expression in CKD supports findings from other patient groups and opens up novel nutritional treatment opportunities in CKD.

Although choline-rich dietary intervention studies have been few, choline supplementation has been shown to reduce inflammatory cytokines, upregulating the mRNA expression of antioxidant enzymes via the Nrf2 pathway [28]. Moreover, He *et al*. [13] have treated VSMCs with choline and observed Nrf2 translocation to the nucleus, with concomitant increase in HO-1 and NQO1 expression and reduction in mitochondrial ROS levels. They have also shown that choline inhibited the production of IL-6. Our findings are commensurate and in accord with these data.

Choline treatment may also be of benefit in mitigating the effects of salt damage in the kidney. In a study to evaluate the effect of salt load on Nrf2 expression in murine kidneys and cortical collecting duct cells (mpkCCD cells), it was observed that Nrf2 expression was reduced in both mice and the cells treated with sodium chloride (NaCl). Interestingly, when

the cells were treated with choline-Cl, Nrf2 expression was unaltered [29]. It is important noticing that the Tolerable Upper Intake Level (UL) according to IOM (1998) is 3.5 g/day of choline for people 19 years and older and high intake may provoke fishy body odor due to high production of TMAO by the gut microbiota, also may cause some gastrointestinal effects such as vomiting [23].

Our findings are clinically relevant from the perspective of the benefits of prescribing a low-protein diet (0.55 g/Kg/day) for patients on conservative treatment which have been recognized to slow the progression of the disease [30]. However, according to the present study's findings, the protein source should be evaluated since this prescription as the reduction of proteins in CKD, especially of animal origin, can lead to choline deficiency and, therefore, can also worsen inflammation and oxidative stress. We suggest that other good sources of choline than animal sources could be prescribed to these patients such as almonds, broccoli and, cauliflower.

In conclusion, our findings of an independent association between choline levels and Nrf2 expression in PBMCs of patients with CKD suggest that nutrients rich in choline may be a novel approach to mitigate the progression of kidney disease and target the uremic phenotype. Until our findings have been confirmed in more extensive studies, this result should be interpreted with caution—the small number of patients and the cross-sectional design limit the study's value.

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Conflict of interest statement

The authors do not declare any potential conflicts of interest.

Contribution of each author

Denise Mafra and Ludmila Cardozo contributed to the data collection and sample analysis, coordinated the study, restructured, writing and revised the manuscript. Marcelo Ribeiro-Alves contributed to the data analysis, produced the figures. Peter Stenvinkel, Paul Shiels and Peter Bergman restructured and revised the manuscript.

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